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(54) Screening test for reverse-transcriptase containing virus.

(57) A single step method of screening blood and other sources for the presence of a reverse-transcriptase-containing virus comprising assaying a sample suspected of containing such a virus for the presence of particle-associated reverse-transcriptase activity.

**EP 0 186 526 A1**

Description

Screening Test For Reverse-Transcriptase  
Containing Virus

Technical Field

5       The present invention relates to a screening test for detecting the presence of reverse-transcriptase containing virus in blood, blood products or in any source containing such virus. More particularly, the present invention relates to the detection and  
10 diagnosis of non-A, non-B hepatitis in blood donors by determining the presence of reverse transcriptase activity in the body fluid, preferably in a blood bank setting. An advantage of the present invention is to prevent transmission of retrovirus related infection  
15 through blood donor (transfusion) program or through plasma-related products by identifying such blood, serum, plasma or products derived therefrom which may be carriers of the retrovirus by using the test disclosed herein.

20 Background Art

Non-A, non-B hepatitis is presumed to be caused by an agent(s) which is serologically distinct from hepatitis A virus and hepatitis B virus. The diagnosis of this disease relies on the serological exclusion of  
25 hepatitis A, hepatitis B, cytomegalovirus, and Epstein-Barr virus.

Non-A, non-B hepatitis infection has been reported worldwide. It accounts for 20% of sporadic cases of

hepatitis among adults. In the United States, this type of hepatitis accounts for 90% of post-transfusion hepatitis. An alarming 50% of these cases develop chronic hepatitis, and such individuals remain as  
5 potential sources of infection.

The existence of a transmissible agent in this disease has been demonstrated. However, presently there is no test to identify the non-A, non-B agent(s). The present invention for the first time  
10 demonstrates that non-A, non-B hepatitis is caused by a retrovirus of retrovirus-like agent and provides a method of screening for the same in a clinical setting, particularly in a blood-bank type program.

#### Disclosure of Invention

15 It is, therefore, an object of the present invention to provide a method for screening blood or blood donors capable of transmitting retrovirus related infection which may be pathogenic.

It is a further object of the present invention to  
20 provide a method of detecting in the blood, serum, plasma or plasma derived products, the presence of virus having reverse transcriptase activity.

It is another object of the present invention to provide a method of detecting the presence of an agent  
25 causing non-A, non-B hepatitis.

It is a still further object of the present invention to provide a kit for the detection of pathogenic or contagious retrovirus, including an agent causing non-A, non-B hepatitis regardless of its

epidemiology.

Other objects and advantages will become apparent as the detailed description of the invention proceeds.

Brief Description Of Drawings

5        These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

10        Figure 1 shows sucrose density gradient banding of inoculum I and localization of reverse transcriptase activity.

Best Mode For Carrying Out The Invention

15        These and other objects and advantages of the present invention are achieved by a screening test for detecting the presence of reverse transcriptase containing viruses in serum or blood, particularly in a blood donor program.

20        The term "blood" as used herein includes not only blood per se but also serum, plasma and any other products or fractions obtained or derived from blood or blood component.

25        Although the screening test described herein detects the presence of reverse transcriptase of whatsoever origin, it should be noted that the reverse transcriptase (RT) in the retroviruses as described herein are particle-associated, i.e., the RT is found

encapsulated within the virus.

Hence, in order to detect the RT, a first essential step is to isolate the virus particles from soluable fraction of the blood. The isolated virus particles are then disrupted or lysed to release the RT therefrom and the specific enzyme activity then assayed.

It should be clear, therefore, that the RT referred herein is specifically of viral origin and not a soluble protein associated with normal parts of the body, tissue or body fluid.

As far as it is known, RT is found associated with all retroviruses tested. Hence, in this sense it may be designated a marker of such viruses. In particular, however, the viruses which belong to this group and which are clinically more significant are the human T-cell lymphocytotropic type I, II and III (HTVB I, II or III) and non-A, non-B hepatitis virus. It may be noted that the present invention is the first to show that non-A, non-B hepatitis is of retroviral origin; hence detectable by RT assay.

It is noted that the term "retrovirus" as used herein includes retrovirus-like agents or entities which have the same density and exhibit RT activity as found in retroviruses mentioned above.

Although any suitable method of detecting RT activity can be used for the practice of this invention, it may be noted that the preferred methods include any radiolabelled enzymatic, histologic, radioimmuno, fluorescent, antigen-antibody, ELISA

(enzyme-linked-immunosorbent assay) and the like. Monoclonal or polyclonal antibodies against purified RT or cleavage products of RT are particularly preferred. Such assay techniques are well known and should be obvious to those of ordinary skill in the art. All references or publications cited hereunder are incorporated herein by reference. Preferred methods and materials are now described.

The abbreviations used herein are as follows:  
NANBH, non-A, non-B hepatitis; RT, reverse transcriptase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PEG, polyethylene glycol 6000; HAV, hepatitis A virus; HBV, hepatitis B virus; RSV, Rous sarcoma virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HTLV III, human T-cell lymphocytotropic virus, type III; AIDS, Acquired Immune Deficiency Syndrome.

#### Materials and Methods

##### Infectious Sera and Plasma-Derived Products

Four serum inocula and two plasma-derived products were studied. Each of them had previously been shown to transmit NANBH to humans and/or to chimpanzees and to be free of HAV, HBV, CMV, and EBV.

Four inocula were sera from patients with NANBH. Inoculum I was a well-defined serum obtained from a patient with chronic NANBH acquired following blood transfusion. The inoculum was known to have transmitted NANBH to another human by accidental inoculation and to chimpanzees. Inocula SE and RP are sera obtained during the acute phase of NANBH from two

patients who both developed chronic NANBH. Inoculum SE is from a patient with blood transfusion-associated NANBH, and inoculum RP is from a male homosexual. Each of these sera transmitted NANBH to chimpanzees which are valid human surrogates for testing purposes. The fourth inoculum (inoculum H) was a well-defined serum obtained from a patient with chronic NANBH which was shown to transmit NANBH to chimpanzees.

Two inocula were plasma-derived products manufactured in the U.S., antihemophilic factor and fibrinogen, respectively. Each of them had been implicated in the transmission of NANBH to patients, and both were shown to transmit NANBH to chimpanzees.

#### NANBH Patients and Health Controls

Serum specimens from 12 patients, each with clinically, histologically, and (by exclusion) serologically confirmed NANBH were studied. These patients included recipients of blood transfusion (3 patients, all of whom developed chronic NANBH), intravenous drug users (4 patients, 3 of whom developed chronic NANBH), and sporadic cases without any known exposure to hepatitis agents (5 patients, 2 of whom developed chronic NANBH). The diagnosis of NANBH was made in each of these patients based upon serum ALT activity (at least 5 times the upper limit of normal, 40 IU/ml), liver histology consistent with viral hepatitis and the absence of serologic markers for HAV (anti-HAV antibodies of the IgM-type), HBV (HBsAg in serum or anti-HBc alone in serum), CMV, or EBV.

Control sera were obtained from 49 healthy individuals, of whom 13 were workers in a plasma

derivatives research and regulatory laboratory and 36 were paid plasmapheresis donors. Both of these groups are known to be at somewhat higher risk of NANBH than the general population.

5 Chimpanzees

Two chimpanzees (Pan troglodytes), 1278 and 1284, were obtained from a breeding colony as described by Tabor et al in Lancet, 1978, 1, 463 and Tabor et al in N. Engl. J. Med, 1980, 303, 140.

10 Detection of Reverse Transcriptase (RT) Activity

RT Assay. Serum samples (100  $\mu$ l), negative control material (fetal bovine serum), and positive control material (Rouse sarcoma virus,  $1 \times 10^{10}$  particles) were all initially centrifuged in 5 ml of  
 15 35% glycerol in 0.5 M Tris-HCl, pH 7.0, at 77,000 x g for 1 hour at 4°C to pellet viral particles from serum proteins as described by Sarngadharan et al in Methods Cancer Res., 1976, 12, 3. The pellet was treated with  
 20 0.25% octylphenol ethylene oxide condensate (Nonidet P40) to disrupt viral particles. Each treated sample was then added to 40  $\mu$ l of solution consisting of 60 mM Tris-HCl, pH 8.3, 8 mM MgCl, 80 mM KCl, 20 mM dithiothreitol, 0.1  $\mu$ g actinomycin D, 80  $\mu$ M each of  
 25 [ $^3$ H]TTP (thymidine triphosphate, specific activity 1.2 Ci/mmol), and 1  $\mu$ g poly(rA) p(dT)<sub>10</sub> as template-primer. The reaction was incubated at 37°C for 1 hour and terminated by adding 40  $\mu$ l of 1 mg/ml yeast tRNA and 5 ml 10% TCA containing 0.2 mM sodium  
 30 phosphophosphate. After 30 minutes, the precipitable radioactivity was collected on glass fiber filters,



dried, and the radioactivity determined by liquid scintillation. The sample was considered positive if the sample cpm were 2 times the negative control cpm (mean  $0.35 \times 10^3$ ). This positive cut-off ( $0.7 \times 10^3$  cpm), based on a titration of RSV particles, represents the RT activity associated with  $1 \times 10^4$  particles of RSV. A gross estimate of virus titer in a given inoculum can, therefore, be obtained by relating the RT activity in cpm associated with various numbers of RSV particles, and comparing the cpm obtained with those for the inoculum. The product assayed is the radiolabelled DNA.

Localization of RT in Sucrose Gradient  
Ultracentrifugation Fractions of Infectious Sera

One ml each of inoculum I and inoculum SE were centrifuged separately in an SW41 rotor by layering on 11 ml of a 10% to 60% (by weight) sucrose gradient made in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA, and centrifuged at 30,000 rpm for 19 hours at 4°C in a Beckman LB-70 ultracentrifuge. Fractions (0.7 ml each) were collected from the bottom of the gradient and the absorbance at 260 nm determined with an LKB UVcord. After removal of sucrose by centrifugation, the fractions were assayed for RT activity as described.

Inoculation of RT Positive Ultracentrifugation  
Fractions into Chimpanzees

Fractions from each individual gradient which contained RT activity were pooled (fractions 12-15 in 2.8 ml), filter-sterilized (0.22  $\mu$ M filter), and injected intravenously into chimpanzees 1278 (inoculum I) and 1280 (inoculum SE). Both chimpanzees were bled

weekly to monitor serum enzyme activities (ALT and AST) and serological markers of hepatitis. In addition, liver biopsies were obtained biweekly and examined by light microscopy for evidence of hepatitis and by  
5 electron microscopy for specific ultrastructural changes in NANBH.

#### Biophysical and Biochemical Characterization of RT

In three serum specimens (inocula I, SE, and RP), the RT activity was assayed following treatment with  
10 6.5% polyethylene glycol 6000 (PEG) according to Welsh et al in Nucleic Acids Res., 1980, 8, 2349 and, in a separate analysis, in the presence of 5 µg RNase A and two different exogenous template-primers as described by Goodman et al in Proc. Natl. Acad. Sci. U.S.A.,  
15 1971, 68, 2203 and Milstein et al in J. Clin. Microbiology, 1975, 1, 353.

#### Results

Particle-associated RT activity was detected in all 6 infectious NANBH materials and in all 12 sera  
20 from patients with acute or chronic NANBH. The RT activity ranged from  $0.85 \times 10^3$  cpm to  $16.6 \times 10^3$  cpm. In contrast, 47 of 49 sera (96%) from healthy controls lacked enzyme activity (CPM ranged from  $0.2 \times 10^3$  to  $0.56 \times 10^3$ ). Sera from two healthy controls  
25 yielded  $1.2 \times 10^3$  and  $1.0 \times 10^3$  cpm, respectively, and were regarded as low positives (cut-off  $0.7 \times 10^3$  cpm). Table I shows the data from these studies.

Table 1  
REVERSE TRANSCRIPTASE (RT) ACTIVITY

Material studied	No. tested	No. positive (%)	RT activity ( $\times 10^3$ cpm) (range and mean)
Sera proven infectious in previous NANBH studies	4	4 (100%)	1.40 <sup>a</sup> - 16.6 mean: 5.95
Plasma-derived products proven infectious in previous NANBH studies	2	2 (100%) mean: 1.08	0.85 - 1.30
Serum samples from NANBH patients obtained during the acute phase of infection	12	12 (100%)	0.86 - 2.70 mean: 1.49
Serum samples from healthy laboratory workers and paid plasmapheresis donors	49	2 ( 4%)	1.0 - 1.20 <sup>b</sup> mean: 1.1

<sup>a</sup> This value represents the RT activity in 100  $\mu$ l of a  $10^{-4}$  dilution of inoculum H.

<sup>b</sup> These values represent the RT activity in the two positive samples. The negative samples have values ranging from  $0.21 \times 10^3$  to  $0.56 \times 10^3$  cpm.

As shown in Fig. 1 peak RT activity for inoculum I banded at 1.14 g/ml in a sucrose gradient. Similar banding patterns were obtained with two other serum inocula. Peak RT activity for inocula SE and RP are also banded at 1.14 g/ml.

Following inoculation of chimpanzees with RT-positive sucrose gradient fractions from either inoculum I (chimpanzee 1278) or inoculum SE (chimpanzee 1284), both animals developed NANBH as confirmed by elevations of serum ALT activity (at least 3 times the baseline level), histologic evidence of hepatitis by light microscopy, and specific ultrastructural cytoplasmic alterations (type C-III tubules) by electron microscopy.

The biophysical and biochemical characteristics of the RT activity detected by the assay described here appear in Table II.

Table II  
NATURE OF THE REVERSE TRANSCRIPTASE (RT) ACTIVITY

Addition <u>b</u> or Treatment		RT activity
		(x 10 <sup>3</sup> cpm/assay)
5	Complete <u>a</u>	4.09
	Actinomycin D, 100 µg	4.01
	6.5% PEG pellet <u>c</u>	4.11
	poly(rA)·p(dT) <sub>10</sub> , 1 µg	7.29
	poly(dA)·p(dT) <sub>10</sub> , 1 µg	3.64
10	poly(rA)·p(dT) <sub>10</sub> , 1 µg, and RNase A, 5 µg	4.34

a The complete system represents the standard RT reaction described in Materials and Methods with the exception of the exogenous template. The activity  
15 represents synthesis using the endogenous template only.

b Addition to the complete reaction described above.

c PEG precipitation of viral particles prior to  
20 addition to complete system.

Besides banding at a discrete density, RT activity is associated with viral particles since it was completely recovered in the 6.5% PEG precipitate. The RT activity showed a preference for poly(rA) p(dT)<sub>10</sub> over poly(dA) p(dT)<sub>10</sub> as template-primer, a feature which distinguishes the viral enzyme from cellular DNA polymerases. Additionally, the incorporation of [<sup>3</sup>H]TTP was insensitive to actinomycin D, which inhibits DNA-dependent DNA synthesis. The viral RT activity with endogenous template-primer was sensitive to RNase A digestion, whereas the reaction with exogenous template-primer, [poly(rA) p(dT)<sub>10</sub>] was unaffected.

The finding of particle-associated RT activity in 4 infectious sera and in 2 infectious plasma-derived products, as well as in 12 serum samples from the acute or chronic phase of NANBH, demonstrated that this disease is caused by a virus or a virus-like agent possessing this enzyme. The location of this RT activity in sucrose gradient fractions (peak activity at 1.14 g/ml) and the transmission of typical NANBH to chimpanzees by inoculating RT-positive sucrose gradient fractions provide evidence that the NANBH agent in the sera and plasma-derived products studied (as well as in the patient sera examined) is a retrovirus or retrovirus-like agent(s). The finding of RT activity in the sera of 2 out of 49 controls which banded at a density consistent with that of retrovirus strongly suggested that these individuals are infected with a retrovirus or retrovirus-like agent. Indeed, all 49 of the controls were at a higher risk for NANBH than the general population.

The finding of RT activity in all 12 sera from patients with different epidemiological types of NANBH indicated that one or more retrovirus or retrovirus-like agent(s) caused all 12 cases of NANBH. The  
5 absence of RT activity in 47 of 49 sera from healthy laboratory workers and paid plasma donors, all of whom are at a higher risk for acquiring NANBH than the general population, support the specificity of the RT assay employed here. Additional evidence for this  
10 specificity is the preference shown for poly(rA) p(dT)<sub>10</sub> as template-primer, the precipitation of RT by PEG, the susceptibility of the endogenous template to inactivation by RNase A, resistance of the reaction product to alkali hydrolysis, and the localization of  
15 the RT activity and infectivity in sucrose gradients at a density consistent with that reported for retrovirus as cited in Sarngadharan et al, supra.

Known characteristics of NANBH and of the agent(s) causing this disease appear to be consistent with the  
20 etiology being a retrovirus or retrovirus-like agent. Inactivation of NANBH agents has been accomplished by formalin, heat, or chloroform, also consistent with their being retroviruses. Chronic infections are common following infection with the NANBH agent(s),  
25 especially those acquired via blood transfusion. Retroviruses characteristically cause chronic infections. Antigen-antibody systems described in association with NANBH are consistent with the development of antibodies to both the external and  
30 internal antigens of retroviruses, all of which appear to coexist with infectious virus in serum. At least one antigen detected by counterelectrophoresis and purified from inoculum I appears to be a glyco-protein similar to one described by Schupbach et al in Science

1984, 224, 503, and present on the surface of the HTLV III retrovirus.

Specific cytoplasmic ultrastructural changes have been consistently seen during NANBH in chimpanzees.

5 Similar alterations have been reported in the lymphocytes of patients with the Acquired Immune Deficiency Syndrome (AIDS), a syndrome which is associated with chronic infection with the retrovirus HTLV III as described by Schaff et al in Lancet 1983,  
10 1, 1336.

Additionally, a known amount of human T-cell lymphocytotropic virus, Type III (HTLV III) when added to human plasma was detected by reverse transcriptase activity. Three products derived from plasma  
15 containing HTLV III, anti-hemophilic factor concentrate, fibrinogen and plasma-protein fraction were also found to contain reverse transcriptase activity: Reverse transcriptase activity was directly related to virus titer, indicating the utility of using  
20 reverse transcriptase activity to estimate the HTLB-III virus titer in plasma.

It is clear from the above that the present invention now makes it possible for blood banks and producers of blood related products to screen all blood  
25 donors and blood products and identify those capable of transmitting retrovirus related pathogenic conditions including NANBH and AIDS. A single screening test utilizing the present invention enables the detection, diagnosis and elimination of retrovirus related  
30 contagious or infectious conditions.



A kit comprising a container containing a suitable RT assay system selected from the group consisting of an enzymatic assay, an antigen-antibody titer assay including mono- or polyclonal antibodies and the like  
5 would be most valuable for clinical and laboratory use in accordance with the present disclosure. Such accessories as micro-titer plates, radiolabelled substrates, pipettes, buffers, coenzymes and the like which are routinely common and well known in the art  
10 are included in the kit in accordance with the present invention.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof  
15 will be suggested to persons skilled in the art are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

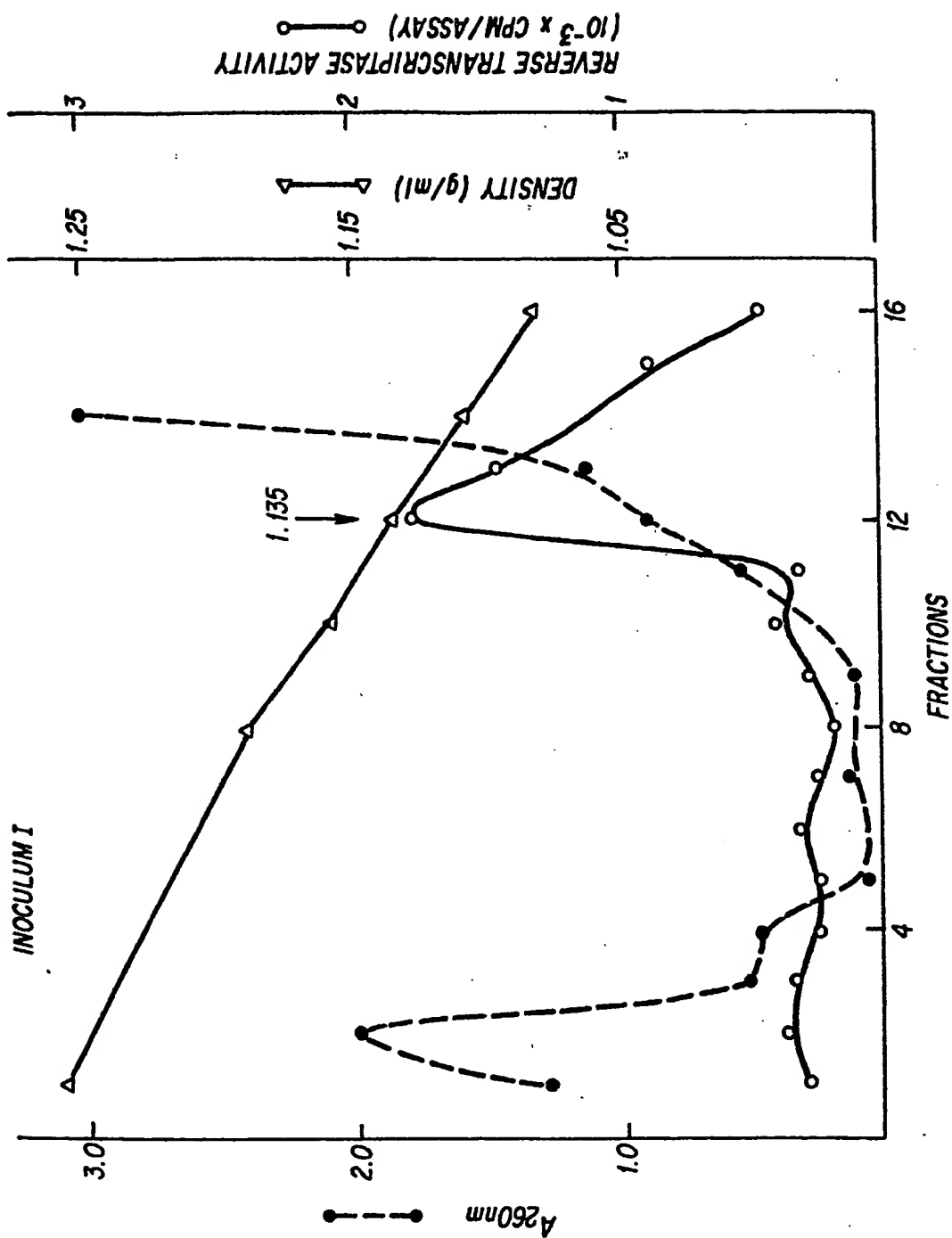
1. A single step method of screening blood for detecting reverse transcriptase containing agent comprising assaying said blood for the presence of  
5 particle associated reverse transcriptase activity.
2. The method of claim 1 wherein said blood comprises serum, plasma or a fraction or product derived therefrom.
3. The method of claim 2 wherein said blood is  
10 obtained from a donor in a blood bank setting.
4. The method of claim 3 wherein said agent is a viral particle.
5. The method of claim 4 wherein said particle is retrovirus.
- 15 6. The method of claim 3 wherein said agent is non-A, non-B hepatitis causing factor.
7. The method of claim 5 wherein said virus is human T-cell lymphocytotropic virus, type III.
8. A method of preventing transmission of  
20 retrovirus caused infection comprising identifying source of reverse transcriptase activity and isolating said source.
9. A method of detecting non-A, non-B hepatitis in a subject comprising assaying reverse transcriptase  
25 activity in a body sample obtained from said subject.

10. A test-kit for detecting the presence of reverse transcriptase containing agent in a sample of blood or blood product comprising means for assaying reverse-transcriptase activity in said sample.

5        11. The test-kit of claim 10 wherein said means is a radiolabelled enzyme assay system.

12. The test-kit of claim 10 wherein said means is an antigen-antibody titer system.

13. The test-kit of claim 10 wherein said means  
10 is a monoclonal antibody system specific against reverse transcriptase.





DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A	CHEMICAL ABSTRACTS, vol. 98, no. 13, 28th March 1983, page 324, column 2, abstract no 103976a, Columbus, Ohio, US; J. WEIS et al.: "Production and characterization of monoclonal antibodies against avian retrovirus reverse transcriptase", & J VIROL. 1983, 45(2), 859-863	1, 13	C 12 Q 1/48 G 01 N 33/573// G 01 N 33/569 G 01 N 33/576 G 01 N 33/577
A	US-A-4 379 839 (S. SPIEGELMAN)  * column 1, line 59 - column 2, line 20 *	1, 2, 4, 5, 10-12	
P, A	US-A-4 520 113 (R.C. GALLO et al.) * column 1 *	1, 7	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
A	EP-A-0 092 249 (EISAI CO., LTD.) * pages 6, 7 *	1, 6	C 12 Q 1/00 G 01 N 33/00
A	WO-A-8 002 598 (USA) * page 2 complete *	2-4, 6	
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 07-02-1986	Examiner GREEN C.H.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons  & : member of the same patent family, corresponding document	



DOCUMENTS CONSIDERED TO BE RELEVANT			Page 2
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P, X	CHEMICAL ABSTRACTS, vol. 102, no. 1, 7th January 1985, page 4020, column 2, abstract no. 4017h, Columbus, Ohio, US; B. SETO et al.: "Detection of reverse transcriptase activity in association with the non-A, non-B hepatitis agent(s)", & LANCET 1984, 2(8409), 941-943  -----	1, 2, 4-6, 8-10	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 07-02-1986	Examiner GREEN C.H.
<b>CATEGORY OF CITED DOCUMENTS</b>			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons  & : member of the same patent family, corresponding document	



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54 Screening test for reverse-transcriptase containing virus.

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March 1983, page 324, column 2, abstract no  
103976a, Columbus, Ohio, US; J. WEIS et al.:  
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CHEMICAL ABSTRACTS, vol. 102, no. 1, 7th  
January 1985, page 4020, column 2, abstract no.  
4017h, Columbus, Ohio, US; B. SETO et al.:

70 Proprietor: **THE UNITED STATES OF AMERICA**  
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56 References cited:  
"Detection of reverse transcriptase activity in  
association with the non-A, non-B hepatitis  
agent(s)", & LANCET 1984, 2(8409), 941-943

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European patent convention).

Courier Press, Leamington Spa, England.

**EP 0 186 526 B1**

**Description****Technical Field**

5 The present invention relates to a screening test for the detection and diagnosis of non-A, non-B hepatitis in blood donors by determining the presence of reverse transcriptase activity in the body fluid, preferably in a blood bank setting. An advantage of the present invention is to prevent transmission of retrovirus related infection through blood donor (transfusion) program or through plasma-related products by identifying such blood, serum, plasma or products derived therefrom which may be carriers of the retrovirus by using the test disclosed herein.

10

**Background Art**

Non-A, non-B hepatitis is presumed to be caused by an agent(s) which is serologically distinct from hepatitis A virus and hepatitis B virus. the diagnosis of this disease relies on the serological exclusion of hepatitis A, hepatitis B, cytomegalovirus, and Epstein-Barr virus.

15

Non-A, non-B hepatitis infection has been reported worldwide. It accounts for 20% of sporadic cases of hepatitis among adults. In the United States, this type of hepatitis accounts for 90% of post-transfusion hepatitis. An alarming 50% of these cases develop chronic hepatitis, and such individuals remain as potential sources of infection.

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The existence of a transmissible agent in this disease has been demonstrated. However, presently there is no test to identify the non-A, non-B agent(s). The present invention for the first time demonstrates that non-A, non-B hepatitis is caused by a retrovirus or retrovirus-like agent and provides a method of screening for the same in a clinical setting, particularly in a blood-bank type program.

**Disclosure of Invention**

25

It is, therefore, an object of the present invention to provide a method for screening blood or blood donors capable of transmitting retrovirus related infection which may be pathogenic.

It is a further object of the present invention to provide a method of detecting in the blood, serum, plasma or plasma derived products, the presence of virus having reverse transcriptase activity.

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It is another object of the present invention to provide a method of detecting the presence of an agent causing non-A, non-B hepatitis.

It is a still further object of the present invention to provide a kit for the detection of pathogenic or contagious retrovirus, including an agent causing non-A, non-B hepatitis regardless of its epidemiology.

Other objects and advantages will become apparent as the detailed description of the invention proceeds.

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**Brief Description of Drawings**

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

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Figure 1 shows sucrose density gradient banding of inoculum I and localization of reverse transcriptase activity.

**Best Mode For Carrying Out The Invention**

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These and other objects and advantages of the present invention are achieved by a screening test for detecting the presence of reverse transcriptase containing viruses in serum or blood, particularly in a blood donor program.

So, the present invention has for object a method for screening a blood composition for the presence of retrovirus particles associated with non-A, non-B hepatitis, which comprises:

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a) isolating said retrovirus particles from a sample of blood composition,

b) disrupting or lysing the isolated retrovirus particles to thereby release the reverse transcriptase therefrom,

c) assaying the reverse transcriptase activity,

d) identifying the NANBH-virus in this reverse transcriptase activity by inoculating reverse transcriptase-positive fractions to chimpanzees,

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e) comparing the NANBH activity assayed in step d) with that assayed in a similarly treated control sample, and

f) concluding the presence of retrovirus particles associated with non-A, non-B hepatitis in said blood composition when the reverse transcriptase activity in the blood composition is about twice or more than that in the control.

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The term "blood" as used herein includes not only blood *per se* but also serum, plasma and any other products or fractions obtained or derived from blood or blood component.

Although the screening test described herein detects the presence of reverse transcriptase of whatsoever origin, it should be noted that the reverse transcriptase (RT) in the retroviruses as described herein are particle-associated, i.e., the RT is found encapsulated within the virus.

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Hence, in order to detect the RT, a first essential step is to isolate the virus particles from soluble



fraction of the blood. The isolated virus particles are then disrupted or lysed to release the RT therefrom and the specific enzyme activity then assayed.

It should be clear, therefore, that the RT referred herein is specifically of viral origin and not a soluble protein associated with normal parts of the body, tissue or body fluid.

5 As far as it is known, RT is found associated with all retroviruses tested. Hence, in this sense it may be designated a marker of such viruses. In particular, however, the viruses which belong to this group and which are clinically more significant are the human T-cell lymphocytotropic type I, II and III (HTVB I, II or III) and non-A, non-B hepatitis virus. It may be noted that the present invention is the first to show that non-A, non-B hepatitis is of retroviral origin; hence detectable by RT assay.

10 It is noted that the term "retrovirus" as used herein includes retrovirus-like agents or entities which have the same density and exhibit RT activity as found in retroviruses mentioned above.

Although any suitable method of detecting RT activity can be used for the practice of this invention, it may be noted that the preferred methods include any radiolabelled enzymatic, histologic, radioimmuno, fluorescent, antigen-antibody, ELISA (enzyme-linked-immunosorbant assay) and the like. Monoclonal or polyclonal antibodies against purified RT or cleavage products of RT are particularly preferred. Such assay techniques are well known and should be obvious to those of ordinary skill in the art. All references or publications cited hereunder are incorporated herein by reference. Preferred methods and materials are now described.

20 The abbreviations used herein are as follows: NANBH, non-A, non-B hepatitis; RT, reverse transcriptase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PEG, polyethylene glycol 6000; HAV, hepatitis A virus; HBV, hepatitis B virus; RSV, Ros sarcoma virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HTLV III, human T-cell lymphocytotropic virus, type III; AIDS, Acquired Immune Deficiency Syndrome.

#### 25 Materials and Methods Infectious Sera and Plasma-Derived Products

Four serum inocula and two plasma-derived products were studied. Each of them had previously been shown to transmit NANBH to humans and/or to chimpanzees and to be free of HAV, HBV, CMV, and EBV.

30 Four inocula were sera from patients from NANBH. Inoculum I was a well-defined serum obtained from a patient with chronic NANBH acquired following blood transfusion. The inoculum was known to have transmitted NANBH to another human by accidental inoculation and to chimpanzees. Inocula SE and RP are sera obtained during the acute phase of NANBH from two patients who both developed chronic NANBH. Inoculum SE is from a patient with blood transfusion-associated NANBH, and inoculum RP is from a male homosexual. Each of these sera transmitted NANBH to chimpanzees which are valid human surrogates for testing purposes. The fourth inoculum (inoculum H) was a well-defined serum obtained from a patient with chronic NANBH which was shown to transmit NANBH to chimpanzees.

35 Two inocula were plasma-derived products manufactured in the U.S., antihemophilic factor and fibrinogen, respectively. Each of them had been implicated in the transmission of NANBH to patients, and both were shown to transmit NANBH to chimpanzees.

#### 40 NANBH Patients and Health Controls

Serum specimens from 12 patients, each with clinically, histologically, and (by exclusion) serologically confirmed NANBH were studied. These patients included recipients of blood transfusion (3 patients, all of whom developed chronic NANBH), intravenous drug users (4 patients, 3 of whom developed chronic NANBH), and sporadic cases without any known exposure to hepatitis agents (5 patients, 2 of whom developed chronic NANBH). The diagnosis of NANBH was made in each of these patients based upon serum ALT activity (at least 5 times the upper limit of normal, 40 IU/ml), liver histology consistent with viral hepatitis and the absence of serologic markers for HAV (anti-HAV antibodies of the IgM-type), HBV (HBsAg in serum or anti-HBc alone in serum), CMV, or EBV.

50 Control sera were obtained from 49 healthy individuals, of whom 13 were workers in a plasma derivatives research and regulatory laboratory and 36 were paid plasmapheresis donors. Both of these groups are known to be at somewhat higher risk of NANBH than the general population.

#### Chimpanzees

55 Two chimpanzees (*Pan troglodytes*), 1278 and 1284, were obtained from a breeding colony as described by Tabor *et al* in Lancet, 1978, 1, 463 and Tabor *et al* in N. Engl. J. Med, 1980, 303, 140.

#### Detection of Reverse Transcriptase (RT) Activity

60 **RT Assay.** Serum samples (100  $\mu$ l), negative control material (fetal bovine serum), and positive control material (Rouse sarcoma virus,  $1 \times 10^{10}$  particles) were all initially centrifuged in 5 ml of 35% glycerol in 0.5 M Tris-HCl, pH 7.0, at  $77,000 \times g$  for 1 hour at  $4^{\circ}\text{C}$  to pellet viral particles from serum proteins as described by Samgadharan *et al* in Methods Cancer Res., 1976, 12, 3. The pellet was treated with 0.25% octylphenol ethylene oxide condensate (Nonidet P40) to disrupt viral particles. Each treated sample was then added to 40  $\mu$ l of solution consisting of 60 mM Tris-HCl, pH 8.3, 8 mM MgCl<sub>2</sub>, 80 mM HCl, 20 mM dithiothreitol, 0.1  $\mu$ g actinomycin D, 80  $\mu$ M each of unlabeled deoxyribonucleoside triphosphate, 20  $\mu$ M

[<sup>3</sup>H]TTP (thymidine triphosphate, specific activity 1.2 Ci/mmol), and 1 µg poly(rA) p(dT)<sub>10</sub> as template-primer. The reaction was incubated at 37°C for 1 hour and terminated by adding 40 µl of 1 mg/ml yeast tRNA and 5 ml 10% TCA containing 0.2 mM sodium phosphophosphate. After 30 minutes, the precipitable radioactivity was collected on glass fiber filters, dried, and the radioactivity determined by liquid scintillation. The sample was considered positive if the sample cpm were 2 times the negative control cpm (mean  $0.35 \times 10^3$ ). This positive cut-off ( $0.7 \times 10^3$  cpm), based on a titration of RSV particles, represents the RT activity with  $1 \times 10^4$  particles of RSV. A gross estimate of virus titer in a given inoculum can, therefore, be obtained by relating the RT activity in cpm associated with various numbers of RSV particles, and comparing the cpm obtained with those for the inoculum. The product assayed is the radiolabelled DNA.

#### Localization of RT in Sucrose Gradient Ultracentrifugation Fractions of Infectious Sera

One ml each of inoculum I and inoculum SE were centrifuged separately in an SW41 rotor by layering on 11 ml of a 10% to 60% (by weight) sucrose gradient made in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA, and centrifuged at 30,000 rpm for 19 hours at 4°C in a Beckman LBR-70 ultracentrifuge. Fractions (0.7 ml each) were collected from the bottom of the gradient and the absorbance at 260 nm determined with an LKB Uvcord. After removal of sucrose by centrifugation, the fractions were assayed for RT activity as described.

#### Inoculation of RT Positive Ultracentrifugation Fractions into Chimpanzees

Fractions from each individual gradient which contained RT activity were pooled (fractions 12—15 in 2.8 ml), filter-sterilized (0.22 µm filter), and injected intravenously into chimpanzees 1278 (inoculum I) and 1280 (inoculum SE). Both chimpanzees were bled weekly to monitor serum enzyme activities (ALT and AST) and serological markers of hepatitis. In addition, liver biopsies were obtained biweekly and examined by light microscopy for evidence of hepatitis and by electron microscopy for specific ultrastructural changes in NANBH.

#### Biophysical and Biochemical Characterization of RT

In three serum specimens (inocula I, SE, and RP), the RT activity was assayed following treatment with 6.5% polyethylene glycol 6000 (PEG) according to *Welsh et al* in *Nucleic Acids Res.*, 1980, 8, 2349 and, in a separate analysis, in the presence of 5 µg RNase A and two different exogenous template-primers are described by *Goodman et al* in *Proc. Natl. Acad. Sci. U.S.A.*, 1971, 68, 2203 and *Milstein et al* in *J. Clin. Microbiology*, 1975, 1, 353.

#### Results

Particle-associated RT activity was detected in all 6 infectious NANBH materials and in all 12 sera from patients with acute or chronic NANBH. The RT activity ranged from  $0.85 \times 10^3$  opm to  $16.6 \times 10^3$  cpm. In contrast, 47 of 49 sera (96%) from healthy controls lacked enzyme activity (CPM ranged from  $0.2 \times 10^3$  to  $0.56 \times 10^3$ ). Sera from two healthy controls yielded  $1.2 \times 10^3$  and  $1.0 \times 10^3$  cpm, respectively, and were regarded as low positives (cut-off  $0.7 \times 10^3$  cpm). Table I shows the data from these studies.

TABLE I  
REVERSE TRANSCRIPTASE (RT) ACTIVITY

Material studied	No. tested	No. positive (%)	RT activity ( $\times 10^3$ cpm) (range and mean)
Sera proven infectious in previous NANBH studies	4	4 (100%)	1.40 <sup>a</sup> —16.6 mean: 5.95
Plasma-derived products proven infectious in previous NANBH studies	2	2 (100%) mean: 1.08	0.8—1.30
Serum samples from NANBH patients obtained during the acute phase of infection	12	12 (100%)	0.86—2.70 mean: 1.49
Serum samples from healthy laboratory workers and paid plasmapheresis donors	49	2 (4%)	1.0—1.20 <sup>b</sup> mean: 1.1

<sup>a</sup> This value represents the RT activity in 100 µl of a  $10^{-4}$  dilution of inoculum H.

<sup>b</sup> These values represent the RT activity in the two positive samples. The negative samples have values ranging from  $0.21 \times 10^3$  to  $0.56 \times 10^3$  cpm.

As shown in Fig. 1 peak RT activity for inoculum I banded at 1.14 g/ml in a sucrose gradient. Similar banding patterns were obtained with two other serum inocula. Peak RT activity for inocula SE and RP are also banded at 1.14 g/ml.

Following inoculation of chimpanzees with RT-positive sucrose gradient fractions from either inoculum I (chimpanzee 1278) or inoculum SE (chimpanzee 1284), both animals developed NANBH as confirmed by elevations of serum ALT activity (at least 3 times the baseline level), histologic evidence of hepatitis by light microscopy, and specific ultrastructural cytoplasmic alterations (type C—III tubules) by electron microscopy.

The biophysical and biochemical characteristics of the RT activity detected by the assay described here appear in Table II.

TABLE II  
NATURE OF THE REVERSE TRANSCRIPTASE (RT) ACTIVITY

15	Addition <sup>b</sup> or Treatment	RT activity
		(× 10 <sup>3</sup> cpm/assay)
20	Complete <sup>a</sup>	4.09
	Actinomycin D, 100 µg	4.01
	6.5% PEG pellet <sup>c</sup>	4.11
25	poly(rA)-p(dT) <sub>10</sub> , 1 µg	7.29
	poly(dA)-p(dT) <sub>10</sub> , 1 µg	3.64
30	poly(rA)-p(dT) <sub>10</sub> , 1 µg, and	4.34
	RNAse A, 5 µg	

<sup>a</sup>The complete system represents the standard RT reaction described in Materials and Methods with the exception of the exogenous template. The activity represents synthesis using the endogenous template only.

<sup>b</sup>Addition to the complete reaction described above.

<sup>c</sup>PEG precipitation of viral particles prior to addition to complete system.

Besides banding at a discrete density, RT activity is associated with viral particles since it was completely recovered in a the 6.5% PEG precipitate. The RT activity showed a preference for poly(rA) p(dT)<sub>10</sub> over poly(dA) p(dT)<sub>10</sub> as template-primer, a feature which distinguishes the viral enzyme from cellular DNA polymerases. Additionally, the incorporation of [<sup>3</sup>H]TTP was insensitive to actinomycin D, which inhibits DNA-dependent DNA synthesis. The viral RT activity with endogenous template-primer was sensitive to RNAse A digestion, whereas the reaction with exogenous template-primer, [poly(rA) p(dT)<sub>10</sub>] was unaffected.

The finding of particle-associated RT activity in 4 infectious sera and in 2 infectious plasma-derived products, as well as in 12 serum samples from the acute or chronic phase of NANBH, demonstrated that this disease is caused by a virus or a virus-like agent possessing this enzyme. The location of this RT activity in sucrose gradient fractions (peak activity at 1.14 g/ml) and the transmission of typical NANBH to chimpanzees by inoculating RT-positive sucrose gradient fractions provide evidence that the NANBH agent in the sera and plasma-derived products studied (as well as in the patient sera examined) in a retrovirus or retrovirus-like agent(s). The finding of RT activity in the sera of 2 out of 49 controls which banded at a density consistent with that of retrovirus strongly suggested that these individuals are infected with a retrovirus or retrovirus-like agent. Indeed, all 49 of the controls were at a higher risk for NANBH than the general population.

The finding of RT activity in all 12 sera from patients with different epidemiological types of NANBH indicated that one or more retrovirus or retrovirus like agent(s) caused all 12 cases of NANBH. The absence of RT activity in 47 of 49 sera from healthy laboratory workers and paid plasma donors, all of whom are at a higher risk for acquiring NANBH than the general population, support the specificity of the RT assay employed here. Additional evidence for this specificity is the preference shown for poly(rA) p(dT)<sub>10</sub> as template-primer, the precipitation of RT by PEG, the susceptibility of the endogenous template to inactivation by RNAse A, resistance of the reaction product to alkali hydrolysis, and the localization of the RT activity and infectivity in sucrose gradients at a density consistent with that reported for retrovirus as cited in Samgadharan et al, *supra*.

Known characteristics of NANBH and of the agent(s) causing this disease appear to be consistent with the etiology being a retrovirus or retrovirus-like agent. Inactivation of NANBH agents has been accomplished by formalin, heat, or chloroform, also consistent with their being retroviruses. Chronic infections are common following infection with the NANBH agent(s), especially those acquired via blood transfusion. Retroviruses characteristically cause chronic infections. Antigen-antibody systems described in association with NANBH are consistent with the development of antibodies to both the external and internal antigens of retroviruses, all of which appear to coexist with infectious virus in serum. At least one antigen detected by counterelectrophoresis and purified from inoculum I appears to be a glyco-protein similar to one described by *Schupbach et al* in *Science* 1984, 224, 503, and present on the surface of the HTLV III retrovirus.

Specific cytoplasmic ultrastructural changes have been consistently seen during NANBH in chimpanzees. Similar alterations have been reported in the lymphocytes of patients with the Acquired Immune Deficiency Syndrome (AIDS), a syndrome which is associated with chronic infection with the retrovirus HTLV III as described by *Schaff et al* in *Lancet* 1983, 1, 1336.

Additionally, a known amount of human T-cell lymphocytotropic virus, Type III (HTLV III) when added to human plasma was detected by reverse transcriptase activity. Three products derived from plasma containing HTLV III, anti-haemophilic factor concentrate, fibrinogen and plasma-protein fraction were also found to contain reverse transcriptase activity: Reverse transcriptase activity was directly related to virus titer, indicating the utility of using reverse transcriptase activity to estimate the HTLV-III virus titer in plasma.

It is clear from the above that the present invention now makes it possible for blood banks and producers of blood related products to screen all blood donors and blood products and identify those capable of transmitting retrovirus related pathogenic conditions including NANBH and AIDS. A single screening test utilizing the present invention enables the detection, diagnosis and elimination of retrovirus related contagious or infectious conditions.

A kit comprising a container containing a suitable RT assay system selected from the group consisting of an enzymatic assay, an antigen-antibody titer assay including mono- or polyclonal antibodies and the like would be most valuable for clinical and laboratory use in accordance with the present disclosure. Such accessories as micro-titer plates, radiolabelled substrates, pipettes, buffers, coenzymes and like which are routinely common and well known in the art are included in the kit in accordance with the present invention.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art are to be included within the spirit and purview of this application and the scope of the appended claims.

### Claims

1. Method for screening a blood composition for the presence of retrovirus particles associated with non-A, non-B hepatitis, comprising:

- a) isolating said retrovirus particles from a sample of blood composition,
- b) disrupting or lysing the isolated retrovirus particles to thereby release the reverse transcriptase therefrom,
- c) assaying the reverse transcriptase activity,
- d) identifying the NANBH-virus in this reverse transcriptase activity by inoculating reverse transcriptase-positive fractions to chimpanzees,
- e) comparing the NANBH activity assayed in step d) with that assayed in a similarly treated control sample, and
- f) concluding the presence of retrovirus particles associated with non-A, non-B hepatitis in said blood composition when the reverse transcriptase activity in the blood composition is about twice or more than that in the control.

2. The method of claim 1 wherein blood comprises serum, plasma or a fraction or product derived therefrom.

3. The method of claim 2 wherein said blood is obtained from a donor in a blood bank setting.

4. A method of detecting non-A, non-B hepatitis in a subject said method comprising assaying reverse transcriptase activity in a body sample obtained from said subject and identifying the NANBH-virus in this reverse transcriptase activity by inoculating reverse transcriptase-positive fractions to chimpanzees.

### Patentansprüche

1. Verfahren zur Reihenuntersuchung einer Blutzusammensetzung auf das Vorhandensein von Retrovirusteilchen, die einer Hepatitis (NANBH), die weder Hepatitis A noch Hepatitis B ist, zugeordnet werden, bei dem

a) die Retrovirusteilchen aus einer Probe der Blutzusammensetzung isoliert werden,

b) die isolierten Retrovirusteilchen zerrissen oder aufgelöst (einer Lysis unterzogen) werden, damit die

Reverse Transkriptase daraus freigesetzt wird,

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- c) die Aktivität der Reversen Transkriptase analysiert wird,  
d) das NANBH-Virus in dieser Aktivität der Reversen Transkriptase durch Einimpfen von Fraktionen, die in bezug auf Reverse Transkriptase positiv sind, in Schimpansen identifiziert wird,  
e) die in Schritt d) analysierte NANBH-Aktivität mit der in einer in derselben Weise behandelten Kontrollprobe analysierten NANBH-Aktivität verglichen wird und  
f) auf das Vorhandensein von Retrovirusteilchen, die einer Hepatitis, die weder Hepatitis A noch Hepatitis B ist, zugeordnet werden, in der Blutzusammensetzung geschlossen wird, wenn die Aktivität der Reversen Transkriptase in der Blutzusammensetzung etwa zweimal so hoch wie in der Kontrollprobe oder höher ist.
2. Verfahren nach Anspruch 1, bei dem das Blut Serum, Plasma oder eine Fraktion oder ein Produkt, das daraus erhalten worden ist, umfaßt.
3. Verfahren nach Anspruch 2, bei dem das Blut von einem Spender in einer Blutbank erhalten worden ist.
4. Verfahren zum Nachweis von Hepatitis (NANBH), die weder Hepatitis A noch Hepatitis B ist, in einem Patienten, bei dem in einer aus dem Körper des Patienten erhaltenen Probe die Aktivität der Reversen Transkriptase analysiert und das NANBH-Virus in dieser Aktivität der Reversen Transkriptase durch Einimpfen von Fraktionen, die in bezug auf Reverse Transkriptase positiv sind, in Schimpansen identifiziert wird.

### 20 Revendications

1. Procédé d'analyse d'une composition de sang pour détecter la présence de particules rétrovirales associées à l'hépatite non-A non-B, dans lequel:
- a) on isole ces particules rétrovirales à partir d'un échantillon de composition de sang;  
b) on éclate ou on lyse les particules rétrovirales isolées afin de libérer ainsi la transcriptase-réverse de celles-ci;  
c) on détermine l'activité de la transcriptase-réverse;  
d) on identifie le virus de la NANBH donnant lieu à cette activité de transcriptase-réverse, en inoculant à des chimpanzés, des fractions transcriptase-réverse-positives;  
e) on compare l'activité NANBH déterminée dans l'étape d) avec celle déterminée dans un échantillon témoin traité de manière analogue; et  
f) on conclut à la présence de particules rétrovirales associées à l'hépatite non-A non-B dans cette composition de sang, lorsque l'activité due à la transcriptase-réverse dans la composition de sang est d'environ deux fois ou plus celle dans le témoin.
2. Procédé selon la revendication 1, dans lequel ce sang comprend du sérum, du plasma, ou une fraction ou un produit dérivé de ce dernier.
3. Procédé selon la revendication 2, dans lequel ce sang est obtenu à partir d'un donneur dans une banque de sang.
4. Procédé de détection de l'hépatite non-A non-B chez un sujet, dans lequel on détermine l'activité due à la transcriptase-réverse dans un échantillon corporel obtenu auprès de ce sujet, et on identifie le virus de la NANBH donnant lieu à cette activité de transcriptase-réverse, en inoculant à des chimpanzés, des fractions transcriptase-réverse-positives.

